

# Branched Chain Amino Acid Metabolism in the Biosynthesis of *Lycopersicon pennellii* Glucose Esters<sup>1</sup>

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## ABSTRACT

*Lycopersicon pennellii* Corr. (D'Arcy) an insect-resistant, wild tomato possesses high densities of glandular trichomes which exude a mixture of 2,3,4-tri-O-acylated glucose esters that function as a physical impediment and feeding deterrent to small arthropod pests. The acyl moieties are branched C<sub>4</sub> and C<sub>5</sub> acids, and branched and straight chain C<sub>10</sub>, C<sub>11</sub>, and C<sub>12</sub> acids. The structure of the branched acyl constituents suggests that the branched chain amino acid biosynthetic pathway participates in their biosynthesis. [<sup>14</sup>C]Valine and deuterated branched chain amino acids (and their oxo-acid derivatives) were incorporated into branched C<sub>4</sub> and C<sub>5</sub> acid groups of glucose esters by a process of transamination, oxidative decarboxylation and subsequent acylation. C<sub>4</sub> and C<sub>5</sub> branched acids were elongated by two carbon units to produce the branched C<sub>10</sub>–C<sub>12</sub> groups. Norvaline, norleucine, allylglycine, and methionine also were processed into acyl moieties and secreted from the trichomes as glucose esters. Changes in the acyl composition of the glucose esters following sulfonylurea herbicide administration support the participation of acetohydroxyacid synthetase and the other enzymes of branched amino acid biosynthesis in the production of glucose esters.

Glandular trichomes and the exudate they produce contribute to insect resistance in a number of plant families. Toxicity, repellence, and physical entrapment have all been demonstrated as modes of action for various trichome exudates (4, 5, 12, 28). The glucose and sucrose esters secreted by glandular trichomes of insect-resistant Solanaceous species have been shown to physically entrap and deter the feeding of small arthropod pests (7, 8, 11, 16, 26). Other results suggest they may also inhibit bacterial and fungal growth (3, 14). Many combinations of short to medium chain fatty acids and patterns of glucose or sucrose esterification are produced by the various species (2, 17–20, 24). Each species produces a characteristic combination of acyl groups esterified to specific positions of the central carbohydrate, but as yet no differences in biological activity have been related to these structural differences (10). The structural diversity of the sugar esters in the *Solanaceae* suggests there is a corresponding diversity of the biochemical pathways responsible for their synthesis. The high biosynthetic activity of some of these pathways is another interesting aspect. *Lycopersicon pennellii* Corr. (D'Arcy) for

example, is known to produce in excess of 400 µg/cm<sup>2</sup> of GE<sup>2</sup> equal to 25% of the leaf dry weight, an enormous commitment of carbon metabolism (6). GE biosynthesis also appears to be very tissue specific. Periclinal chimeric plants consisting of epidermis derived from *L. pennellii* and mesophyll and vascular tissue from *L. esculentum* (which does not synthesize GE) produce GE in similar amounts and of similar composition to *L. pennellii* (DS Walters, unpublished data). These data indicate that GE biosynthesis, if not confined to the trichomes themselves, must take place somewhere in the epidermal layer of cells.

The type IV glandular trichomes of *L. pennellii* exude a complex mixture of 2,3,4 tri-O-acyl glucose compounds (2). The predominant fatty acids that occur in this mixture are 2-methylpropanoic, 2-methylbutanoic, *n*-decanoic, and 8-methylnonanoic acids with lesser but significant amounts of 3-methylbutanoic, 9-methyldecanoic, and *n*-dodecanoic acids (Table I, column 1).

The carbon skeletons of the acyl groups in *L. pennellii* GE suggest their possible derivation from branched chain amino acids (Val, Leu, and Ile) in accordance with Kolattukudy's (21) demonstration of the biosynthesis of branched cuticular waxes from Val and Leu precursors. The acyl moieties found in GE are identical with those produced by the ubiquitous branched chain oxoacid dehydrogenase complex which oxidatively decarboxylates the α-keto-derivatives of branched chain amino acids to form acyl CoA thioesters (9, 30). The branched medium chain (C<sub>10</sub>–C<sub>12</sub>) acyl groups found in *L. pennellii* GE may also be derived from branched chain amino acids via the elongation of branched, short chain acyl CoA primers by a fatty acid synthetase system. Both the chain length of the acyl groups produced and the chain extension of branched acyl CoA primers distinguish this fatty acid synthetase system from that of lipid acyl biosynthesis in primary metabolism.

Based on existing knowledge of amino acid and fatty acid metabolism and the structures and composition of the acyl groups present in *L. pennellii* GE, we propose the biosynthetic pathway for GE shown in Figure 1. The proposed pathway integrates amino acid metabolism and fatty acid biosynthesis. Esterification of the acyl groups to glucose completes the synthesis and may be the only step unique to this class of compounds. Our objective in this paper was to verify the

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<sup>2</sup> Abbreviations: GE, glucose ester; oxo-Val, 2-keto-3-methylbutanoic acid; oxo-Leu, 2-keto-4-methylpentanoic acid; oxo-Ile, 2-keto-3-methylpentanoic acid; ALS, acetohydroxyacid synthetase [EC 4.1.3.18].

proposed pathway using both  $^{14}\text{C}$ -labeled and stable isotopes of branched chain amino acids and their keto derivatives. A preliminary account of these studies has been reported elsewhere (29).

## MATERIALS AND METHODS

### Chemicals

Met, allyl glycine, norvaline, norleucine, oxo-Val, and oxo-Leu were purchased from Sigma. Oxo-Ile was obtained from Fluka,  $[\text{U}^{14}\text{C}]\text{Val}$  from Amersham,  $[\text{H}_8]\text{Val}$  from Cambridge Isotope Laboratories, and  $[\text{H}_{10}]\text{Leu}$  from MSD Isotopes. Technical grade chlorsulfuron and sulfometuron methyl were a gift from Prof. Joseph DiTomaso, Cornell University.

Deuterated  $\alpha$ -keto acids were prepared by an acid-catalyzed exchange reaction in  $^2\text{H}_2\text{O}$  (Aldrich) resulting in deuteration of the  $\beta$ -carbon. The  $\alpha$ -keto acid (600 mg) and 60  $\mu\text{L}$  of thionyl chloride were dissolved in 1 mL of  $^2\text{H}_2\text{O}$ . The reaction mix was refluxed overnight and the water was subsequently removed using a rotary evaporator.

### Precursor Administration

The excised leaves used in these experiments were obtained from *Lycopersicon pennellii* plants maintained in greenhouse facilities of the Department of Entomology, Cornell University. Supplemental lighting was provided by 1000 W metal halide lamps. Terminal branch tips were removed from the plants and placed in deionized water while they were being prepared for precursor incubations. Prior to precursor administration, compound leaves from the third or fourth node

away from the apical meristem were removed and briefly immersed (2–3 s) with agitation in 8 to 10 mL of 100% EtOH to remove preexisting epicuticular GE. This trichome exudate sample was saved for comparison with the fatty acid composition of the same leaf following incubation with one of the putative precursors. The EtOH was immediately shaken from the leaf followed by blotting and evaporation of any remaining solvent using air from a blower (room temperature).

Following exudate removal, a diagonal cut was made on the leaf petiole and the leaf was immediately placed in a deionized  $\text{H}_2\text{O}$  solution containing a potential precursor. The precursor solution reservoir consisted of a plastic Petri dish lid with a parafilm cover stretched over it. Holes were punched in the parafilm for insertion of 2 to 4 leaf petioles and addition of 20 mL of the incubation solution. The leaves were illuminated continuously with a 100 W incandescent lamp 16 inches from the leaf surface. The precursors were administered as 5 mM solutions. Chlorsulfuron and sulfometuron methyl were administered similarly as 10  $\mu\text{M}$  solutions. Incubations proceeded for 48 h during which time deionized water was added to replenish transpirational losses. Following incubation the leaves were rinsed again in 8 to 10 mL of 100% EtOH.

Incubation with  $[\text{U}^{14}\text{C}]\text{Val}$  was performed using a slightly altered technique. The labeled amino acid was administered to the excised leaf petiole in a 100  $\mu\text{L}$  aqueous solution in a polypropylene microcentrifuge tube. After uptake of the solution was complete, two or three 100- $\mu\text{L}$  aliquots of water were sequentially added to the tube to insure that as much of the label as possible had entered the petiole. The leaf was then removed and incubated under incandescent light in deionized  $\text{H}_2\text{O}$ .

### Product Analysis

The ethanol extracts were evaporated to dryness and redissolved in 2 to 3 mL of  $\text{MeCl}_2$ . GLC and GC-MS analysis was performed on the ethyl esters formed from transesterification of the sugar ester fatty acids. An appropriate aliquot (usually 50–100  $\mu\text{L}$ ) was removed to a reaction vial, evaporated and redissolved in 50  $\mu\text{L}$  of heptane. A 20% solution of sodium ethoxide in EtOH was added (20  $\mu\text{L}$ ) and the transesterification allowed to proceed for 5 min at room temperature. The reaction mix was washed with a saturated NaCl solution (50  $\mu\text{L}$ ) to remove unreacted sodium ethoxide as well as free sugar. The vial was vortexed, the aqueous layer was discarded, and the organic layer was washed again with saturated NaCl. The remaining heptane layer was then analyzed immediately by GLC and/or GC-MS.

The fatty acid composition of the sugar esters was determined by gas chromatography of the ethyl esters using a 30 m, 0.24 mm i.d., DB-FFAP (J & W Scientific; Folsom, CA) capillary column. The chromatograph was operated in a backpressure regulated mode (21 psi, He carrier) with a temperature program from 36 to 220°C at 10°/min with a 5 min hold at the initial temperature. A Varian 3700 instrument equipped with an FID was used for routine GLC analysis. GC-MS analysis was performed using the same column and a similar temperature program. The gas flow rate was reduced to compensate for the vacuum effect at the column outlet. A

**Table 1.** *L. pennellii* GE Acyl Group Composition and Relative Incorporation of Deuterated Valine and Leucine into the Various Acyl Groups

First column represents the average percent contribution (and standard errors-16 plants) of acyl groups in *L. pennellii* GE. Second and third columns are percentage of labeled compound recovered relative to its unlabeled homolog following precursor administration. Values are the average of two incubations.

Fatty Acid	Contribution	$[\text{H}_8]\text{Val}$	$[\text{H}_{10}]\text{Leu}$
	%		
Short chain composition			
2-Methyl propanoic	68.9 (0.8)	11.9	— <sup>a</sup>
2-Methyl butanoic	23.2 (0.5)	—	—
3-Methyl butanoic	7.9 (0.4)	44.7	121.6
Medium chain composition			
7-Methyl octanoic	<0.5	+ <sup>b</sup>	+
8-Methyl nonanoic	56.4 (0.8)	8.2	—
<i>n</i> -Decanoic	28.6 (0.6)	—	—
9-Methyl decanoic	2.2 (0.1)	24.6	109.8
<i>n</i> -Undecanoic	<0.5	—	—
10-Methylundecanoic	1.4 (0.1)	+	—
<i>n</i> -Dodecanoic	11.2 (0.2)	—	—
% Contribution of medium chain relative to total acyl groups	38.5 (0.8)		

<sup>a</sup> No detectable incorporation. <sup>b</sup> Incorporation detected but not quantified due to low levels of both labeled and unlabeled compound.

Hewlett-Packard model 5890 GLC in conjunction with an H-P model 5970 Mass Selective Detector and an H-P model 59970C Data Station were used for some of the mass spectral analyses while an equivalent Mass Selective Detector and Data Station coupled with a Carlo Erba model HRGC 5160 chromatograph were used for the remainder. The interface between the capillary column outlet and the mass spectrometer was direct in both instances. Mass scans were performed at a rate of 2/s. Spectra of unlabeled ethyl esters were virtually identical with published spectra.

Isolation of glucose ester bands from TLC plates was performed to verify that novel fatty acids were incorporated into GE rather than extracted from the leaves as free acids (Merck, Silica Gel 60, 0.2 mm thickness plates; EtOAc/Pyridine/H<sub>2</sub>O/AcOH [105:45:15:7.5] solvent system). Sugar ester bands were identified using a carbohydrate spray reagent (diphenylamine/aniline/acetone) on adjacent control lanes (25). Bands were scraped, eluted with EtOH and MeCl<sub>2</sub>, and analyzed by the previously described GLC and GC-MS techniques.

## RESULTS AND DISCUSSION

### <sup>14</sup>C Valine Incorporation

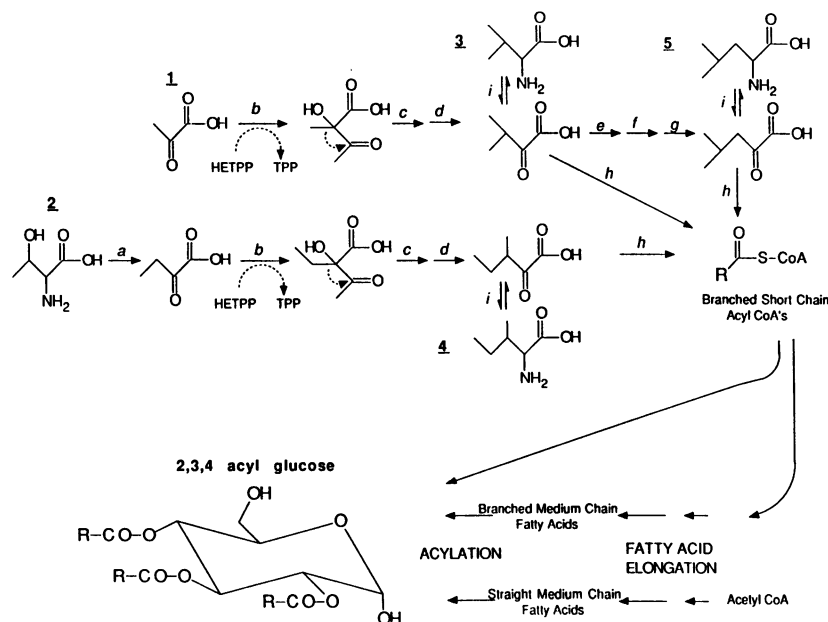
Administration of [U-<sup>14</sup>C]Val to excised leaves resulted in a high rate of incorporation of <sup>14</sup>C into GE. Of a total  $1.1 \times 10^7$  cpm administered to two excised *Lycopersicon pennellii* leaves,  $1.3 \times 10^6$  cpm were recovered in the EtOH wash of the leaf exterior following a 24 h incubation. Scintillation counting of bands recovered from TLC autoradiography of the EtOH wash showed nearly 10% of the total administered activity comigrated with the GE band. An aliquot of the extract was transesterified to fatty acid ethyl esters and trapped from the GLC capillary column outlet by passing the outlet flow through liquid N<sub>2</sub>-cooled Pasteur pipettes packed with Tenax adsorbent. The radioactivity coeluted from the column with esters of 2-methylpropanoic, 3-methylbutanoic, and 8-

methylnonanoic acids (with 63, 10, and 27%, respectively, of the trapped radioactivity). These results are consistent with the transamination of Val to oxo-Val, conversion of oxo-Val to oxo-Leu and the subsequent oxidative decarboxylation and esterification of both keto-acids to glucose (Fig. 1). Extension/elongation of isobutyryl-CoA (the product of oxo-Val oxidative decarboxylation) to 8-methylnonanoate accounts for label incorporation in that compound. The very high level of incorporation encouraged us to attempt administration of stable isotope labeled precursors to enable us to establish precise locations of heavy atoms within the labeled product by GC-MS.

### Stable Isotope Incorporation

Incorporation of deuterated, branched amino acid precursors into branched chain GE acyl groups was extremely good, an indication of the active nature of this anabolic pathway. The amount of deuterated product exceeded (146%) that of the unlabeled material present in some instances, an impressive result considering the substantial amount (10–20%) of preexisting material that remains on the leaf surface following the preincubation EtOH wash mentioned earlier. Because of their mol wt differences, unlabeled ethyl esters and deuterated homologs were resolvable by capillary GLC (Fig. 2) (monodeuterated and dideuterated products were not completely resolved from their unlabeled homologs). Extractions of leaves following both [<sup>2</sup>H<sub>8</sub>]Val and [<sup>2</sup>H<sub>10</sub>]Leu incubations revealed peaks not present in preincubation extracts representing deuterated fatty acid esters which eluted 10 to 15 s before identical unlabeled esters. The isotopic compounds were distinct from their unlabeled counterparts in both GLC-FID and GC-MS analyses.

Figures 2 and 3 illustrate the pattern of [<sup>2</sup>H<sub>8</sub>]Val incorporation and the resulting changes in the mass spectra of the labeled GE acyl groups. The GLC trace in panel 2A shows



**Figure 1.** Outline of proposed pathway of sugar ester biosynthesis in *L. pennellii*. Compounds: (1) pyruvate, (2) Thr, (3) Val, (4) Ile, (5) Leu; HETPP, hydroxyethylthiamine pyrophosphate; TPP, thiamine pyrophosphate. Enzymes: (a) threonine dehydratase, (b) acetohydroxyacid synthase, (c) acetohydroxyacid reductoisomerase, (d) dihydroxyacid dehydratase, (e)  $\alpha$ -isopropylmalate synthase, (f) isopropylmalate isomerase, (g)  $\beta$ -isopropylmalate dehydrogenase, (h) branched chain oxo-acid dehydrogenase, (i) aminotransferase.

the ethyl esters of labeled 2-methylpropanoate, 3-methylbutanoate, 7-methyloctanoate, 8-methylnonanoate, 9-methyldecanoate, and 10-methylundecanoate incorporated into the glucose esters of *L. pennellii* from a [ $^2\text{H}_8$ ]Val precursor. Deuterated 2-methylpropanoate represented 47% of the total labeled acyl groups present following [ $^2\text{H}_8$ ]Val incubation, with 3-methylbutanoate, 8-methylnonanoate, and 9-methyldecanoate contributing 26, 20, and 4% of the total, respectively. Deuterated 7-methyloctanoate and 10-methylundecanoate were detected in only trace amounts (<0.5%). The pattern of incorporation of deuterated Val agrees reasonably well with the incorporation observed for [ $^{14}\text{C}$ ]Val when the much greater amount of precursor administered in the stable isotope experiment (25.0  $\mu\text{mol}$  deuterated Val versus  $8.7 \times 10^{-3} \mu\text{mol}$  [ $^{14}\text{C}$ ]Val per excised leaf) and the extremely inefficient trapping of the labeled compounds from the GLC column outlet are taken into account. The stable isotope experiment was much more sensitive and was able to detect incorporation into minor components while the  $^{14}\text{C}$  experiment only detected incorporation in the three most abundant products. Mass spectra of the unlabeled ethyl-2-methylpropanoate peak and the [ $^2\text{H}_7$ ]ethyl-2-methylpropanoate peak are reproduced

in panels 2B and 2C. [ $^2\text{H}_7$ ]2-Methylpropanoate exhibits an  $\text{M}^+$  ion at  $m/z$  123 as well as ions at  $m/z$  105, 78, and 50 that are paralleled by ions at  $m/z$  116, 101, 71, and 43 in the unlabeled 2-methylpropanoate spectrum. The ion at  $m/z$  101 in the unlabeled 2-methylpropanoate spectrum is produced by the loss of a methyl group ( $\text{M}^+ - 15$ ) and is a common fragment seen in other ethyl esters analyzed. The  $m/z$  105 peak of the deuterated product results from the analogous loss of a deuterated methyl group ( $\text{M}^+ - 18$ ). The fragment ion at  $m/z$  43 arises in part from the isopropyl group in the 2-methylpropanoate alkyl chain and is largely replaced by a peak at  $m/z$  50 in the [ $^2\text{H}_7$ ] product. No  $m/z$  50 peak is present in the unlabeled compound. Loss of the ethoxy group produces ions at  $m/z$  71 and  $m/z$  78 in the undeuterated and deuterated fatty acid, respectively.

Deuterated 3-methylbutanoate produced from [ $^2\text{H}_8$ ]Val incorporation exhibits ion fragments consistent with the unscrambled incorporation of the Val alkyl chain. Figure 3 shows the mass spectra of both the unlabeled and [ $^2\text{H}_7$ ]-3-methylbutanoate. The shift of the base peak from  $m/z$  88 to  $m/z$  89 is the result of the substitution of a deuterium on carbon 3 such that McLafferty rearrangement results in the

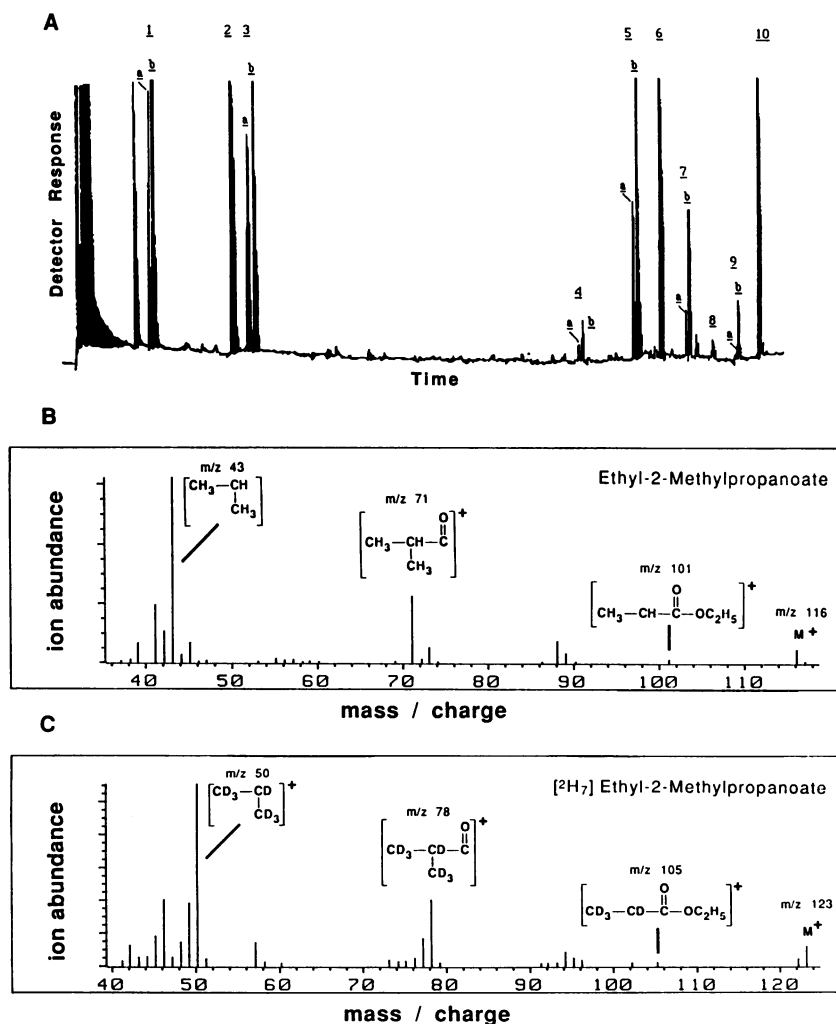


Figure 2. A, Incorporation of [ $^2\text{H}_8$ ]Val in *L. pennellii* glucose esters: GLC-FID trace of acyl constituents of trichome exudate. Peaks are: (1) 2-methylpropanoate, (2) 2-methylbutanoate, (3) 3-methylbutanoate, (4) 7-methyloctanoate, (5) 8-methylnonanoate, (6) *n*-decanoate, (7) 9-methyldecanoate, (8) *n*-undecanoate, (9) 10-methylundecanoate, (10) *n*-dodecanoate. Peaks designated 'a' are deuterated products, 'b' are unlabeled compounds. B, Mass spectrum of peak 1a-unlabeled ethyl 2-methylpropanoate. C, mass spectrum of peak 1b-[ $^2\text{H}_7$ ] ethyl 2-methylpropanoate resulting from [ $^2\text{H}_8$ ]Val incorporation.

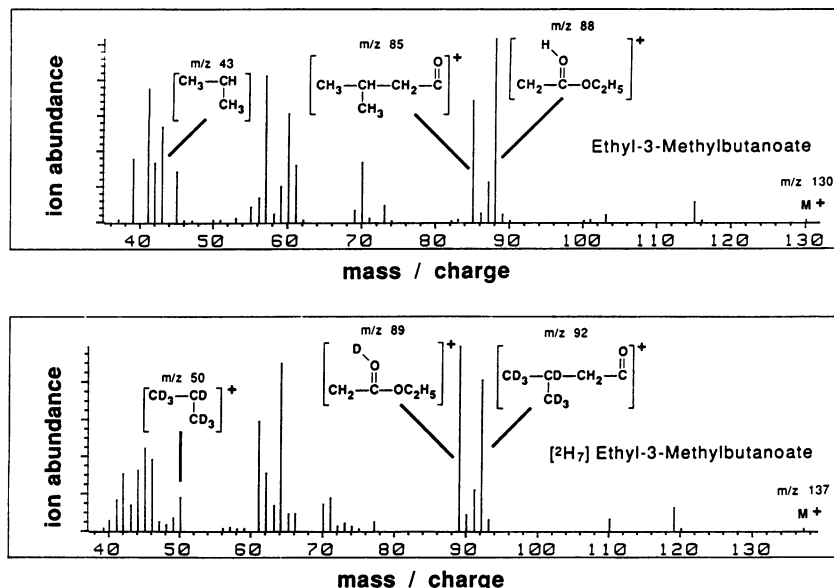


Figure 3. Mass spectrum of peak 3a (Fig. 2)-unlabeled ethyl 3-methylbutanoate. Mass spectrum of peak 3b (Fig. 2)- $[\text{2H}_7]$  ethyl 3-methylbutanoate resulting from  $[\text{2H}_8]$ Val incorporation.

shift of a deuteron rather than the proton shift of the unlabeled compound. The peak at  $m/z$  85 in the unlabeled compound is a common ester fragment,  $\text{M}^+-45$  (loss of ethoxy in this case) to produce the  $\text{R-C=O}^+$  ion. The same ion in the  $[\text{2H}_7]$  compound is found at  $m/z$  92. Loss of a methyl group produces an ion fragment at  $\text{M}^+-15=115$  in the unlabeled compound while the loss of a corresponding deuterated methyl group in the  $[\text{2H}_7]$  compound produces an  $\text{M}^+-18=119$  fragment. The ion at  $m/z$  43 includes the isopropyl fragment in the unlabeled compound but is greatly reduced in  $[\text{2H}_7]$ -3-methylbutanoate and replaced by a significant peak at  $m/z$  50 (deuterated isopropyl fragment).

The straight chain elongated acyl groups exhibited no evidence of incorporation from any of the labeled branched chain amino acids or oxo-acid derivatives. In contrast, the branched  $\text{C}_{10}$ ,  $\text{C}_{11}$ , and  $\text{C}_{12}$  acyl groups show clear evidence of Val incorporation. Deuterated 8-methylnonanoate, 9-methyldecanoate, and 10-methylundecanoate resulting from  $[\text{2H}_8]$ Val incorporation have  $\text{M}^+$  ions 7 mass units above their unlabeled counterparts ( $m/z$  207, 221, and 235, respectively). Ions at  $\text{M}^+-29$ ,  $\text{M}^+-43$ , and  $\text{M}^+-45$  also occur 7 mass units higher in the deuterated compounds than in the unlabeled compounds. A diminished ion at  $m/z$  43 and the appearance of a significant new peak at  $m/z$  50 in all three compounds is indicative of a  $[\text{2H}_7]$ -labeled terminal isopropyl group as described for the short chain acids. This is strong evidence for the unscrambled incorporation of the branched alkyl chain of  $[\text{2H}_8]$ Val into the branched elongated acyl groups.

Deuterated Leu fed to excised leaves of *L. pennellii* was also metabolized to GE acyl groups in a manner consistent with the biosynthetic scheme of Figure 1.  $[\text{2H}_{10}]$ Leu was incorporated into  $[\text{2H}_9]$ 3-methylbutanoate and its elongation products, primarily 9-methyldecanoate (Table I). Not surprisingly however, a small amount of  $[\text{2H}_9]$ -labeled 7-methyloctanoate was also detected, consistent with the proposed elongation of the short chain precursor by 2-carbon units. The mass spectra of these labeled fatty acids exhibit similar patterns to

those described for the  $[\text{2H}_8]$ Val products. The isotopic Leu products produce molecular ions 9 mass units higher than unlabeled compounds (3-methylbutanoate  $\text{M}^+=139$ , 9-methyldecanoate  $\text{M}^+=223$ , 7-methyloctanoate  $\text{M}^+=195$ ). While both  $[\text{2H}_9]$  compounds produce the same series of hydrocarbon clusters at  $m/z$  101, 115, 143, and 157 (157 does not occur in 7-methyloctanoate for obvious reasons) as the unlabeled compound, they also exhibit  $\text{M}^+-29$ ,  $\text{M}^+-43$ , and  $\text{M}^+-45$  ions indicative of their isotopic composition. Once again the diminished  $m/z$  43 containing the isopropyl ion and a new significant  $m/z$  50 ion indicates isotopic incorporation at the end of the alkyl chain.

The incubation of *L. pennellii* leaves with  $[\text{2H}_8]$ Val resulted in GE containing  $[\text{2H}_7]$ 2-methylpropanoic,  $[\text{2H}_7]$ 3-methylbutanoic,  $[\text{2H}_7]$ 8-methylnonanoic, and  $[\text{2H}_7]$ 9-methyldecanoic acids (Table I). In addition small amounts of  $[\text{2H}_7]$ 10-methylundecanoic and  $[\text{2H}_7]$ 7-methyloctanoic acid moieties were also detected. This is consistent with the alternative routes of acyl group biosynthesis from a Val precursor presented in Figure 1, namely: 1) oxidative decarboxylation of oxo-Val to isobutyryl-CoA followed by direct esterification to glucose; 2) processing of oxo-Val to oxo-Leu (via the three enzymes of Leu biosynthesis:  $\alpha$ -isopropylmalate synthase, isopropylmalate isomerase, and  $\beta$ -isopropylmalate dehydrogenase) followed by oxidative decarboxylation to isovaleryl-CoA and esterification; and 3) elongation of isobutyryl and isovaleryl-CoA by fatty acid synthetase followed by esterification.

The hypothesis that branched chain acyl groups of the glucose esters of *L. pennellii* trichome exudate are produced from branched amino acids via oxoacid intermediates was further supported by incubations with deuterated oxo-Ile and oxo-Leu, the transamination products of Ile and Leu, respectively. GC-MS of fatty acids resulting from the  $[\text{2H}_2]$ oxo-Leu incubation revealed an isotopic shoulder at the leading edge of the GLC peaks of the two expected products, 3-methylbutanoate and elongated 9-methyldecanoate. The spectra of the  $[\text{2H}_2]$ 3-methylbutanoate product contained the following

abundant ions:  $m/z$  132 ( $M^+$ ),  $m/z$  90 (B), and major peaks at  $m/z$  117, 105, 90, 87, 72, 62, and 59 that parallel corresponding peaks in the unlabeled spectra, 2 mass units lower in every instance. At the low end of the spectrum, however, the isopropyl peak at  $m/z$  43 remains unchanged, appropriate for a compound deuterated at carbon 3 in the oxoacid precursor. Deuterated 9-methyl decanoate exhibits a molecular ion at 216 and fragment ions at  $M^+-29$ ,  $M^+-43$ , and  $M^+-45$  ( $m/z$  187, 173, and 171) that compare with corresponding ions at  $m/z$  214, 185, 171, and 169 in the unlabeled compound. A minor peak indicating the presence of trace amounts of deuterated 7-methyloctanoate was also observed suggesting elongation by the addition of acetate units.

Results of the  $[^2H_1]$ oxo-Ile incubation demonstrated that 2-methylbutanoate is readily synthesized from oxo-Ile but that 2-methylbutyryl-CoA is a much poorer substrate for the fatty acid synthetase elongation system than the other branched acids. Following incubation, isotopic labeling was detected in both 2-methylbutanoate and 8-methyldecanoate, its elongation product. Isotopically labeled  $[^2H_1]$ 2-methylbutanoate exhibited a molecular ion at  $m/z$  131 and fragment ions at  $m/z$  116, 103, 86, and 58 which occur 1 mass unit higher than the same ions in the unlabeled spectrum. In the elongated 8-methyldecanoate product, a molecular ion at  $m/z$  215 and fragments at 186 and 158 are present in the deuterated compound and parallel ions 1 mass unit lower in the unlabeled 8-methyldecanoate. Only trace amounts of labeled 8-methyldecanoate were present in comparison to a much greater contribution from 2-methylbutanoate. Under normal conditions, this compound does not make a meaningful contribution ( $<0.5\%$ ) to the fatty acid composition of the GE of *L. pennellii*. However, the detection of label incorporation in this experiment provides supporting evidence for the use of short chain primers in the synthesis of the medium chain acyl groups and indicates that while 2-methylbutanoate can be elongated like the other branched acyl groups, it is apparently elongated at a much slower rate.

The fact that *L. pennellii* leaves incorporate  $[^2H_8]$ Val into deuterated 3-methylbutanoate at nearly the level which Val is incorporated into deuterated 2-methylpropanoate (60–70% as much) is likely the result of conversion of Val to Leu by amino acid metabolism in regions of the leaf distinct from the epidermal site of GE synthesis. Thus, the deuterated precursor arrives at the site of GE synthesis as deuterated Leu rather than deuterated Val. This also explains the increase in the contribution of 3-methylbutanoate in leaves fed unlabeled Val. Apparently, Val fed to excised leaves is converted to Leu

more efficiently by metabolic pathways in subepidermal leaf tissue than occurs at the usual site of GE acyl biosynthesis in the epidermis.

### Atypical Fatty Acid Incorporation

Other amino acids were also effectively metabolized by excised *L. pennellii* leaves to produce GE containing atypical acyl groups. A summary of these precursors and their primary products is given in Table II. Incubation with norvaline resulted in the production of sugar esters with *n*-butanoate as significant acyl components (5% of the total short chain acids). In the absence of this precursor, *n*-butanoate is not present in detectable amounts. Even carbon chain elongation products, *n*-decanoate and *n*-dodecanoate, were also present in correspondingly increased abundance following norvaline administration. Thus, *n*-butanoate can be both elongated and directly esterified in the GE biosynthetic process. These results also indicate that *n*-decanoate and *n*-dodecanoate are not synthesized from an *n*-butanoate precursor under ordinary conditions in the plant. If such a precursor were present, one would expect to find *n*-butanoate as a significant acyl component of the GE given the amounts of *n*-decanoic and *n*-dodecanoic acid present.  $C_{10}$ – $C_{12}$  straight chain acyl groups are therefore probably derived from acetate by *de novo* fatty acid biosynthesis.

Excised *L. pennellii* leaves also processed norleucine into sugar ester components in a manner consistent with the proposed pathway. A 1-carbon loss through oxidative decarboxylation followed by either direct esterification or elongation then esterification predicts the occurrence of *n*-pentanoic and *n*-undecanoic acids in GE following norleucine incubation. Following norleucine incubation, *n*-pentanoate comprised an average of 20% of the short chain fatty acids in *L. pennellii* GE and *n*-undecanoate represented as much as 18% of the total medium chain acids. *n*-Nonanoic acid was also present in the GE following norleucine incubation. As shown in Table I, these straight chain odd carbon number fatty acids are present in only trace or nondetectable amounts in the intact plant.

Branched chain oxo-acid dehydrogenase in mammals has been shown to process a variety of  $\alpha$ -keto acids including the oxoacid derivative of Met (2-oxo-methylthiobutanoate) (15). It might be expected then that Met could be processed into methylthiopropionate and incorporated into GE. Although the amount of methylthiopropionate recovered was fairly low in comparison to some of the other atypical products (2–4%

**Table II.** Incorporation of Unlabeled and Atypical Amino Acids into Glucose Esters of *L. pennellii* Trichome Exudate

Precursor	Major Product <sup>a</sup>	Elongation Products
Norvaline	<i>n</i> -Butanoate (9%)	<i>n</i> -Decanoate, <i>n</i> -dodecanoate
Norleucine	<i>n</i> -Pentanoate (41%)	<i>n</i> -Nonanoate, <i>n</i> -undecanoate
Methionine	Methylthiopropionate (3%)	None
Allyl glycine	5-Hexenoate (19%)	5-Hexenoate
Threonine	2-Methylbutanoate (13%)	8-Methyldecanoate

<sup>a</sup> Mean amount of product relative to isobutyrate (100%).

of the total medium chain acids), *L. pennellii* was able to metabolize Met into GE acyl groups. The mass spectrum of the sulfide-containing acid is shown in Figure 4. The molecular ion at 148 and the major peaks at 61 and 74 possess the characteristic  $M+2$  peaks of appropriate abundance (4.4%) for a sulfur containing compound. The strong  $m/z$  61 fragment is diagnostic of a primary methyl sulfide while  $m/z$  103 represents the  $M^+-45$  peak expected from an ethyl ester. Ions at  $m/z$  74 and 75 probably represent fragments resulting from cleavage between the carbonyl and the  $\alpha$ -carbons, a prominent cleavage in short chain esters that might be favored by the presence of a sulfide group. Like the other ethyl esters that were analyzed, this compound has an  $M^+-29$  fragment ( $m/z$  119) representing the loss of the ethyl group from the alcohol portion of the ester.

The processing of allyl glycine resulted in synthesis of GE containing an unsaturated acyl group. Perhaps even more interesting, the sole product recovered was 5-hexenoic acid, the result of a single acetate addition. An  $M^+$  ion at  $m/z$  142, parent peak at  $m/z$  69 and prominent fragments at  $m/z$  41, 127, and 197 are consistent with ethyl 5-hexenoate, the structure resulting from transamination, oxidative decarboxylation, and 1 cycle of acetate elongation of allyl glycine. No products beyond a 6 carbon chain were produced, likely the result of an interaction between the terminal double bond and an active site of the fatty acid synthetase complex causing a premature termination of elongation. The lack of detectable incorporation of 3-butenate may be an indication of a lack of acceptance by the acyltransferase.

Incubation of excised *L. pennellii* leaves with Thr further emphasized the link between the branched chain fatty acid biosynthetic pathway and amino acid biosynthesis. Thr is known to be metabolized to oxo-Leu by the action of four enzymes, three of which also catalyze the conversion of pyruvate to Val (Fig. 1) (1). Thr administration resulted in an increase in the relative amount of 2-methylbutanoate present in short chain fatty acids of GE from the 21 to 23% range of untreated plants to levels of 30 to 40%.

#### Effect of Acetohydroxyacid Synthetase Inhibition on Glucose Ester Composition

An important result from our investigation was the effect of acetohydroxyacid synthetase [ALS; EC 4.1.3.18] inhibitors on the GE fatty acid composition of *L. pennellii*. ALS catalyzes the condensation of two pyruvate molecules in Val synthesis, or one pyruvate and one  $\alpha$ -ketobutyrate molecule in Ile biosynthesis (Fig. 1, enzyme *b*). Sulfonyleurea, imada-

zolinone, and triazolo pyrimidine herbicides are selective inhibitors of this enzyme (22). Administration of two different ALS inhibitors, chlorsulfuron, and sulfometuron methyl at 10  $\mu$ M concentrations both produced a substantial increase in the amount of straight chain  $C_9$  and  $C_{11}$  fatty acids as well as significant quantities of propanoic acid (up to 8% of the short chain acids). While trace amounts of *n*-nonanoic and *n*-undecanoic acids are detectable in untreated *L. pennellii* leaves, each was recovered in amounts equivalent to 2 to 8% of the total medium chain acids following ALS inhibition. Propanoic acid, however, was not detected in any of the previous analyses of GE from *L. pennellii* trichome exudate.

Production of sugar esters containing significant amounts of propanoate, *n*-nonanoate, and *n*-undecanoate supports the hypothesis that ALS catalyzes the metabolic step for branched fatty acid synthesis shown in Figure 1. Inhibition of this enzyme blocks the conversion of Thr to Ile and results in the buildup of  $\alpha$ -ketobutyrate. Since we have already demonstrated that Met, norleucine, norvaline, and allyl glycine can be processed into fatty acid sugar esters via their oxoacid counterparts, it is not surprising that  $\alpha$ -ketobutyrate could be similarly metabolized resulting in the production of propionyl-CoA which is both esterified directly and also elongated to straight, odd chain acids. The accumulation of  $\alpha$ -ketobutyrate and propionyl-CoA has been shown to result from sulfometuron methyl inhibition of ALS in bacteria (23, 27).

#### CONCLUSION

The enzymes responsible for GE biosynthesis appear to have been recruited from three primary metabolic pathways; amino acid metabolism, fatty acid biosynthesis, and carbohydrate metabolism. In amino acid catabolism,  $\alpha$ -keto acids formed by transamination of their amino acid analogs are oxidatively decarboxylated to form acyl-CoA thioesters one carbon shorter than the precursor. The same enzymatic steps, in conjunction with elongation by a fatty acid synthetase system, are used in the biosynthesis of the GE acyl groups. The fatty acid synthetase system involved in GE biosynthesis differs from the system responsible for membrane lipid biosynthesis in both the termination of elongation at the  $C_{10}$ - $C_{12}$  level and the acceptance of branched chain acyl CoA primers to produce branched elongated acyl groups. Esterification of the sugar moiety appears to be the only enzymatic step unique to this pathway, but the enzyme(s) that catalyzes the acyltransferase reaction may also be derived from enzymes that perform similar reactions in primary metabolism, such as glycerolipid biosynthesis.

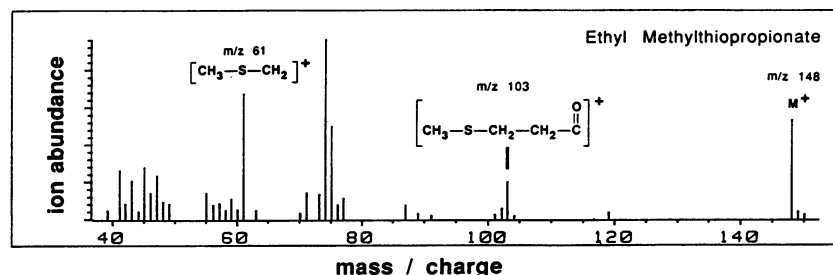


Figure 4. Mass spectrum of methylthiopropionate in glucose esters of *L. pennellii* following methionine incubation.

Based on knowledge of lipid and amino acid metabolism, it is probable that acyl-ACP and/or acyl-CoA compounds are the acyl group donors in glucose ester formation. Acylation reactions in glycerolipid biosynthesis are known to use both thioesters as substrates (13). In this paper, we have demonstrated the oxidative decarboxylation of a number of branched chain precursors prior to their incorporation into GE. Because the products of branched chain oxo-acid dehydrogenase complex are acyl-CoAs we believe it is likely that acyl CoA thioesters are the primary acyl donors in GE biosynthesis.

The change in acyl group composition in response to sulfonylurea herbicides indicates that acetohydroxyacid synthetase is an integral part of the GE biosynthetic pathway and that the enzyme is similar to the one involved in amino acid biosynthesis. The degree of similarity between this and other enzymes responsible for GE biosynthesis and those involved in primary metabolism will be interesting to compare in future work.

#### Note Added in Proof

L. Kandra, R. Severson, and G. J. Wagner ([1990] Eur J Biochem 188: 385) have recently proposed a similar branched-chain amino acid origin for the acyl constituents of tobacco sucrose esters.

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